CMV-Specific TCR-Transgenic T Cells for Immunotherapy

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CMV-Specific TCR-Transgenic T Cells for Immunotherapy

Andrea Schub,* Ingrid G. Schuster,† Wolfgang Hammerschmidt,* and Andreas Moosmann2*

Reactivation of CMV can cause severe disease after allogeneic hemopoietic stem cell transplantation. Adoptive T cell therapy was successfully used for patients who had received transplants from CMV-positive donors. However, patients with transplants from CMV-negative donors are at highest risk, and an adoptive therapy is missing because CMV-specific T cells are not available from such donors. To address this problem, we used retroviral transfer of CMV-specific TCR genes. We generated CMV-specific T cell clones of several HLA restrictions recognizing the endogenously processed Ag pp65. The genes of four TCRs were cloned and transferred to primary T cells from CMV-negative donors. These CMV-TCR-transgenic T cells displayed a broad spectrum of important effector functions (secretion of IFN-γ and IL-2, cytotoxicity, proliferation) in response to endogenously processed pp65 and could be enriched and expanded by strictly Ag-specific stimulation. Expansion of engineered T cells was accompanied by an increase in specific effector functions, indicating that the transferred specificity is stable and fully functional. Hence, we expect these CMV-TCR-transgenic T cells to be effective in controlling acute CMV disease and establishing an antiviral memory. The Journal of Immunology, 2009, 183: 6819–6830.

Materials and Methods
The standard cell culture medium was RPMI 1640 (Invitrogen) supplemented with 10% FCS (PAA Laboratories), 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen), and 100 nM sodium selenite (ICN Biochemicals). 293T cells were cultured in DMEM (Invitrogen) with the same supplements.

Cells and cell lines
PBMCs from healthy donors (Table I) were collected with donors’ informed consent following the requirements of the local ethical board and the principles expressed in the Helsinki Declaration. PBMCs were obtained by centrifugation on Ficoll/Hypaque (Biochrom). HLA typing was performed by PCR-based methods (IMGM). Stably pp65-expressing mini-lymphoblastoid cell lines (mLCL) and control (pp65-negative) mLCLs...
were generated by infection of PBMCs with B cell-transforming mini-EBV vectors (32). CD40-activated B-blast (BBL) cultures were established as described (33) and maintained by weekly replating PBMCs on irradiated (140 Gy) murine fibroblasts, stably expressing the human CD40L, in the presence of 2 ng/ml IL-4 (R&D Systems). The TCR<sup>α</sup>-deficient T cell lines Jurkat76 (J76) (34) and J76 stably expressing the human CD8α chain (J76CD8; kindly provided by W. Uckert, Max-Delbrück-Center, Berlin, Germany) were used as recipient cells for TCR transfer studies. 293T cells were used for packaging of the retroviral vectors.

**Generation of CMV-specific T cell clones**

We generated T cells against the following HLA class I-restricted epitopes from pp65: NLV/VPMVATV, an 495–503, HLA-A*0201-restricted (abbreviated NLV); IPSINVIHY, aa 123–131, HLA-B*3501-restricted (IPS); YSEHPTFAQY, aa 363–373, HLA-A*0101-restricted (YSE).

YSE-specific T cells from donor 7 (Table I) were reactivated within PBMCs by stimulation with the irradiated (50 Gy) autologous pp65-expressing mLCLs (32). Per well of a 12-well plate, 6 × 10<sup>4</sup> PBMCs and 1.5 × 10<sup>6</sup> pp65-expressing mLCLs were cocultivated in 3 ml of medium. On day 10 and then every 7 days, cells were pooled, counted by trypan blue staining, and replated at 3 × 10<sup>4</sup> cells/3 ml of medium per well, adding fresh irradiated (50 Gy) pp65 mLCL as stimulators at an effector-stimulator ratio of 3:1 and 100 U/ml rIL-2. After addition of 1 ml of retrovirus-containing supernatant, the plates were spinoculated for 2 h at 800 × g and 32°C. J76 and J76CD8 cells (10<sup>6</sup> in 1 ml), which had been activated with 50 U/ml rIL-2 and 50 ng/ml anti-CD3 Ab (OKT-3, kindly provided by E. Kremmer, Helmholtz Zentrum, Munich, Germany) 2 days earlier, were transduced in 24-well plates precoated with 5 μg/ml RetroNectin (Takara) with 4 μg/ml prostate serum (MP Biomedicals) and 100 U/ml rIL-2. After addition of 1 ml of retrovirus-containing supernatant, the plates were spinoculated for 2 h at 800 × g and 32°C. J76 and J76CD8 cells (10<sup>6</sup> in 1 ml) were transduced as described above but without adding rIL-2. Medium was replaced after 24 h.

**Restimulation of TCR-transduced cells**

Weekly restimulation of transduced PBMCs was started 6 days after TCR transfer. For Ag-specific stimulation, transduced T cells (1.5 × 10<sup>5</sup> ml/well) were cocultivated with irradiated (50 Gy) autologous pp65-expressing mLCL at a ratio of 4:1 with 100 U/ml rIL-2 in 24-well plates. For nonspecific stimulation, transduced T cells (1.5 × 10<sup>5</sup>/1.5 ml/well) were cocultivated with 1.5 × 10<sup>6</sup> per well of a mixture of irradiated (50 Gy) allogeneic PBMCs from three different donors and 1.5 × 10<sup>5</sup>/well irradiated (50 Gy) autologous pp65-negative mLCL with 100 U/ml rIL-2 and 50 ng/ml anti-CD3 Ab (OKT-3). Medium was replaced after 24 h.

**Flow cytometry**

Multimer staining was performed by incubating the T cells for 10 min at room temperature with PE-labeled HLA/peptide tetramer or unlabeled HLA/peptide pentamer. The cells were counterstained on ice for 15 min with anti-CD4-FITC, anti-CD3-PE-Cy5, and anti-CD8-APC Abs (all BD Pharmingen), and, in the case of unlabeled pentamers, with Pro5 Fluorotag R-PE (Proimmune). Directly after staining, the cells were fixed by 1.6% formaldehyde (Carl Roth). As control, T cells were stained as explained above but without adding the respective multimer. PE-labeled NLV/ A*0201 tetramer was purchased from Beckman Coulter; unlabeled IPS/ B*3501 and YSE/A*0101 pentamers were purchased from Proimmune. Cells were analyzed on a BD Biosciences FACSCalibur flow cytometer. Data analysis was performed using FlowJo 8.8.4 software (Tree Star). For analysis, viable lymphocytes were gated in a forward-sideward scatter dot plot. All shown dot plots span, on both coordinates, a range from 1 to 10,000 arbitrary units of fluorescence intensity in a logarithmic scale.

**T cell effector assays**

CMV-specific T cell clones and TCR-transduced PBMCs were analyzed for cytokine secretion by ELISA. Effector cells (10<sup>6</sup>) were cocultivated overnight with target cells (2 × 10<sup>5</sup>) in 200 μl/well of a 96 V-well plate at 37°C and 5% CO2. Then supernatants were harvested, and IFN-γ and IL-2 chain genes into the vector MP71Gpre (MP71-TCRα and MP71-TCRβ) replacing the GFP gene. The GFP-encoding MP71Gpre vector was used as control to evaluate infection efficiencies. All TCR cassettes used in this study were verified by DNA sequencing (Sequiseq).

**Retroviral TCR transfer**

To produce amphotropic MLV-pseudotyped retroviruses, 293T cells were cotransfected with calcium phosphate precipitation with expression plasmids encoding the Moloney MLV gag/pol genes (pCDNA3.1MLVgp/p) and the MLV-10A1 env gene (pALF-10A1) together 1) with the respective TCR-encoding retroviral vector plasmids MP71-TCRα and MP71-TCRβ, or 2) with the GFP-encoding plasmid MP71Gpre (transfection and transduction control), or 3) without retroviral vector plasmids (mock control) (41). Forty-eight hours after transfection, the retroviral supernatant was harvested, filtered (0.45 μm pore size), and used directly for infection of PBMCs, J76 cells, and J76CD8 cells. PBMCs (10<sup>6</sup> in 1 ml), which had been activated with 50 U/ml rIL-2 and 50 ng/ml anti-CD3 Ab (OKT-3, kindly provided by E. Kremmer, Helmholtz Zentrum, Munich, Germany) 2 days earlier, were transduced in 24-well plates precoated with 5 μg/ml RetroNectin (Takara) in the presence of 4 μg/ml protease salt (MP Biomedicals) and 100 U/ml rIL-2. After addition of 1 ml of retrovirus-containing supernatant, the plates were spinoculated for 2 h at 800 × g and 32°C. J76 and J76CD8 cells (10<sup>6</sup> in 1 ml) were transduced as described above but without adding rIL-2. Medium was replaced after 24 h.

**Table I. HLA types and virus carrier state of donors**

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Neg, Negative; Pos, positive; N.D., not determined.

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ELISAs were performed according to the manufacturer’s recommendations (Mabtech).

Cytotoxicity of TCR-transduced PBMCs was analyzed by calcein release assay as described previously (33).

Results

Generation of CMV pp65-specific T cell clones

We generated CMV-specific CD8\(^+\) T cell clones against the epitopes YSE (HLA-A*0101), NLV (HLA-A*0201), and IPS (HLA-B*3501) derived from pp65. CMV-specific T cells were enriched by Ag-specific stimulation of primary T cells from three different CMV-seropositive donors. NLV- and IPS-specific T cells were obtained by stimulating PBMCs with the epitope peptide, as shown for NLV-specific T cells in Fig. 1A. YSE-specific T cells were enriched by repeated stimulation with the autologous pp65-expressing mLCL (Fig. 1B). Subsequently, T cell clones were generated by limiting dilution.

To test whether the T cell clones recognize pp65 Ag when intracellularly expressed, processed and presented, we analyzed their reactivity to pp65-expressing mLCLs, control (pp65-negative) mLCLs, and CD40-activated BBLs from various donors. For characterization of their TCR, we selected T cell clones which strongly and exclusively recognized autologous or HLA-matched pp65-expressing mLCLs, as shown for NLV- and IPS-specific clones (Fig. 1, D and E). All T cell clones with this reaction pattern showed a clear staining with the corresponding HLA/peptide multimer (Fig. 1C).

CMV-specific TCRs of these T cell clones were characterized by TCR subfamily-specific PCR (Table II). The NLV-specific T cell clones derived from donor 6 used different TRAV and TRBV genes. The TCR NLV2 from T cell clones JG-9 and JG-24 is a public TCR and was also found in NLV-specific T cell clones of another donor not included in this study. In addition, other groups described NLV-specific T cells from various donors which used the same TCR \(\alpha\)- or \(\beta\)-chain or closely related variants (25, 26, 30, 31). In contrast, the TCRs NLV3, YSE, and IPS have not been described thus far. We found the CDR3\(\alpha\) region of the NLV3-TCR to be extremely short; this TCR might recognize Ag by adopting an unusual structure, meriting further investigation.

Retroviral transfer of CMV-specific TCRs into TCR\(\alpha\)-\(\beta\)-deficient T cell lines

The TCR \(\alpha\)- and \(\beta\)-chain genes derived from these four CMV-specific T cell clones were individually inserted into the retroviral vector plasmid pMP71Gpre, and infectious retroviral supernatants were produced. To investigate transgenic TCR expression in the absence of an endogenous TCR, we transferred the CMV-specific TCRs to TCR\(\alpha\)-\(\beta\)-deficient J76 cells and to J76CD8 cells stably expressing the human CD8\(\alpha\) chain (Fig. 2A). All TCRs were expressed on J76CD8 cells and were strongly stained with the respective HLA/peptide multimer. Staining levels on J76 cells without CD8\(\alpha\) were reduced, but three of the four TCRs could be detected on these cells, except for the IPS-TCR. We conclude that the TCRs NLV2, NLV3, and YSE did not strictly require CD8 to bind their target HLA/peptide complex.

Transfer of CMV-specific TCRs into primary T cells of CMV-seronegative donors

To confer CMV-specific reactivity on primary T cells from CMV-negative donors, we performed retroviral transfer of our CMV-specific TCRs. Four days after transfer, all TCRs were expressed on PBMCs and could be stained with peptide/HLA multimers, whereas mock-transduced cells were multimer negative (Fig. 2, B and C). Multimer-staining levels of the individual TCRs were comparable between different donors with different HLA backgrounds (Table I), but the levels varied among the four different TCRs (Fig. 2C). Proportions of multimer-positive cells were similar within different T cell subsets (CD3\(^+\), CD8\(^+\), CD4\(^+\)) except for the IPS-TCR. This TCR was not detectable on CD4\(^+\)CD8\(^+\) T cells, suggesting CD8 dependency, which is consistent with our previous observation that this TCR could not be detected on J76 cells (Fig. 2A). The general transduction efficiency, as determined with GFP-expressing retrovirus, was comparable for all donors (45–60%). To check whether the TCR-transgenic T cells had acquired reactivity against pp65-expressing cells already at this early stage, we cocultivated the transduced PBMCs with autologous, allogeneic HLA-matched and HLA-mismatched target cells, namely pp65-expressing and pp65-negative mLCLs and BBLs, at day 6 after TCR transfer, and determined the amount of released IFN-\(\gamma\) and IL-2. NLV2-TCR-transduced as well as NLV3-TCR-transduced PBMCs specifically produced considerable amounts of IFN-\(\gamma\) (Fig. 2D) and some IL-2 (Fig. 2E) after coculture with HLA-A2-positive pp65-expressing mLCLs. Cocultivation with BBLs (pp65 and EBV negative) did not induce IFN-\(\gamma\), but low amounts of IFN-\(\gamma\) were released when the TCR-transduced cells were tested against pp65-negative mLCLs, indicating the presence of some EBV-specific memory T cells, which was expected because the donor (donor 1) was EBV positive (Table I).

These results show that CMV-specific T cells recognizing endogenously processed Ag can be rapidly produced by TCR transfer in a simple 8-day procedure from PBMCs from seronegative donors.

Simultaneous expansion and enrichment of TCR-transgenic T cells by Ag-specific stimulation

For successful T cell therapy, TCR-transgenic T cells must be able to recognize the endogenously processed Ag and proliferate in an Ag-specific manner in vivo to achieve sufficient function and maintenance after therapeutic T cell transfer. Therefore, we investigated whether CMV-TCR-transduced T cells could be expanded and enriched by Ag-specific stimulation. As Ag-specific stimulators, we used the autologous pp65-expressing mLCL, which presents endogenously processed pp65 epitopes on class I and II HLA molecules (22, 32). For comparison, we performed nonspecific stimulation with anti-CD3 Ab, a mix of allogeneic PBMCs derived from three unrelated donors, and the autologous mLCL without pp65 expression. The stimulation protocol is schematically shown in Fig. 3A.

For all four CMV-TCRs, CMV-TCR-transgenic CD8\(^+\) T cells were specifically enriched by stimulation with the endogenously processed CMV Ag pp65 (Fig. 4C). The same was true if only CMV-TCR-transgenic CD8\(^+\) T cells were evaluated (Fig. 4D). Examples of multimer stainings are shown in Fig. 3B. As before, for a given TCR proportions of TCR-transgenic T cells were similar among different PBMC donors (Fig. 4, C and D). Ag-specific stimulation led to an expansion of each TCR-transduced T cell culture in terms of absolute cell numbers (Fig. 4A). For the two NLV-TCRs, specific and nonspecific stimulation were comparable in terms of total cell expansion (Fig. 4A). For the IPS- and YSE-TCRs, nonspecific stimulation led to the strongest overall cell expansion (Fig. 4A), but only specific stimulation raised the proportion of TCR-transgenic T cells (Fig. 4, C and D). Both stimulation protocols preferentially expanded CD8\(^+\) T cells (Fig. 4B).

From these data, we determined the increase in absolute numbers of TCR-transgenic CD8\(^+\) T cells (expansion) as well as the
FIGURE 1. Establishment of CMV pp65-specific polyclonal T cell lines and CD8⁺ T cell clones. A, The frequency of CMV-specific CD8⁺ T cells recognizing the HLA-A*0201-restricted epitope NLV was assessed by staining with NLV tetramer and anti-CD8 Ab at days (d) 0, 6, and 14 after initial stimulation of PBMCs from donor 6 with NLV peptide. As a control, the staining procedure was performed without adding NLV tetramer. B, The frequency of CMV-specific CD8⁺ T cells recognizing the HLA-A*0101-restricted epitope YSE was assessed by staining with YSE-pentamer and anti-CD8 Ab at days 10, 22, and 29 during repeated stimulation of PBMCs from donor 7 using autologous pp65-expressing mLCL. As a control, the staining procedure was performed without adding YSE pentamer. C, Multimer binding of the CMV-specific CD8⁺ T cell clones JG-24, JG-33, BF-33, and MD-19 was analyzed by staining with the respective HLA-peptide multimer (abbreviated NLV, IPS, YSE) and anti-CD8 Ab. As controls, the staining procedure was performed without adding the respective multimer. D, Release of IFN-γ by the CMV-specific CD8⁺ T cell clones JG-24 (NLV2-TCR) and JG-33 (NLV3-TCR) after recognition of endogenously presented pp65 was analyzed by ELISA. JG-24 T cells (■) or JG-33 T cells (□) were tested against autologous, allogeneic HLA-A2-positive, or allogeneic HLA-A2-negative target cells (donors are indicated in brackets). Mean values and ranges of duplicates are shown. E, Release of IFN-γ by the CMV-specific CD8⁺ T cell clone BF-33 (IPS-TCR) after recognition of endogenously presented pp65 was analyzed by ELISA. BF-33 T cells were tested against autologous, allogeneic HLA-B*3501-positive, or allogeneic HLA-B*3501-negative target cells (donors are indicated in brackets). Values are mean values and ranges of duplicates.
increase in the proportion of TCR-transgenic CD8\(^+\) T cells (enrichment), after both Ag-specific and nonspecific stimulation (Table III). For each TCR, both stimulation conditions led to an impressive expansion of CD8\(^+\) T cells expressing the transgenic TCR (36- to 672-fold; Table III). With the exception of the IPS-TCR-transgenic T cells, Ag-specific stimulation resulted in a superior expansion compared with nonspecific stimulation. For all four TCRs, enrichment of TCR-transgenic CD8\(^+\) T cells was

<table>
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<tr>
<th>Donor</th>
<th>T Cell Clone</th>
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<th>β-Chain</th>
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<td></td>
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<td></td>
<td>BV CDR3(β) BJ</td>
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<td>12-4<em>01 CAS SSANYGY TFG 1–2</em>01</td>
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<td>11-2<em>01 CAS SADSNGEL FFG 2–2</em>01</td>
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**FIGURE 2.** Transfer of four different CMV-specific TCRs into T cell lines and PBMCs. A, The CMV pp65-specific TCRs NLV2, NLV3, IPS, and YSE were transferred into J76 cells (top) and J76CD8 cells (bottom). Expression of the introduced TCR was analyzed by staining with the respective HLA-peptide-multimer (thick line) at day 4 after transfer. As control, mock-transduced cells were analyzed by staining with the same multimer (thin line). Numbers indicate the percentage of multimer-positive cells. B, PBMCs from CMV-seronegative donors were transduced with the CMV pp65-specific TCRs NLV2 (donor 1), NLV3 (donor 1), IPS (donor 3), and YSE (donor 3; bottom), or mock-transduced (top). Expression of the introduced TCR was analyzed by staining with the respective HLA-peptide multimer at day 4 after transfer. C, The TCRs NLV2, NLV3, IPS, and YSE were transferred into PBMCs from different CMV-seronegative donors, and the frequency of multimer-positive cells within different T cell subsets, CD3\(^+\) cells (black bars), CD8\(^+\)CD4\(^-\) cells (gray bars), and CD8\(^+\)CD4\(^+\) cells (light gray bars), was determined by costaining with the respective HLA/peptide-multimer and Abs against CD3, CD4, and CD8 at day 4 after TCR transfer. D and E, CMV-specific reactivity of TCR-transgenic T cells against cells endogenously presenting the target Ag pp65 was analyzed by IFN-γ ELISA (D) and IL-2 ELISA (E) at day 6 after TCR transfer. NLV2 (black bars), NLV3 (dark gray bars), or mock-transduced (light gray bars) PBMCs from donor 1 were tested against autologous, allogeneic HLA-A2-positive, or allogeneic HLA-A2-negative target cells (donors are indicated in brackets). Mean values and ranges of duplicates are shown. The percentage of multimer-positive cells at day 4 after transfer is indicated.
stronger after specific stimulation than after nonspecific stimulation (Table III). Together, these data show that endogenously processed Ag stimulates a robust and specific expansion and enrichment of T cells carrying transgenic CMV-specific TCRs.

CD4+ T cells expressing these HLA class-I-restricted TCRs were maintained (NLV2, NLV3) or even expanded (YSE) by specific stimulation, except for the IPS-TCR (Fig. 4E), which was the only TCR that could not be detected on J76 cells lacking CD8 (Fig. 2A).

We observed no great variations over time in the intensity of multimer staining of gated multimer-positive populations during specific or nonspecific stimulation, as shown for NLV2-TCR-transduced cells from two donors in Fig. 3C, suggesting that TCR expression levels were relatively stable.

Release of effector cytokines after Ag-specific vs nonspecific expansion of CMV-TCR-transgenic T cells

To check whether CMV-TCR-transgenic T cells had maintained their specific function after Ag-specific expansion, we reassessed their pp65-specific cytokine release after three rounds of stimulation. Ag-specifically or nonspecifically stimulated TCR-transduced cultures were cocultivated with a panel of target cells endogenously expressing pp65 and HLA-mismatched or pp65-negative controls (Fig. 5). Compared with the situation 6 days after TCR transfer (Fig. 2, D and E), we found strongly increased pp65-specific release of IFN-γ for each CMV-TCR-transduced T cell line after Ag-specific expansion (Fig. 5, A and B). After nonspecific expansion, secretion was much lower (Fig. 5, A and B), consistent with lower numbers of multimer-positive cells (Fig. 4C). HLA-mismatched and pp65-negative controls were only weakly recognized. As expected, mock-transduced T cell cultures showed no pp65-specific reactivity.

Additionally, TCR-transduced T cell cultures specifically secreted considerable amounts of IL-2 in response to endogenously processed pp65 after Ag-specific stimulation (Fig. 5C). In contrast, there was no CMV-specific release of IL-2 by nonspecifically stimulated cultures.

These results show that Ag-specific expansion significantly enhanced the specific function of the TCR-transduced T cells (Fig. 5), consistent with the increase in CMV multimer-positive T cells (Fig. 4). To more precisely quantify the stability of Ag-specific function of TCR-transgenic T cells, we determined the amount of IFN-γ secreted by one multimer-positive T cell before and after three rounds of stimulation, under the assumption that only multimer-positive T cells will specifically secrete IFN-γ upon challenge with CMV APCs. As shown for NLV2-transduced cells in Fig. 6, the amount of cytokine secreted per multimer-positive cell in response to the CMV mLCL was 12-fold higher after Ag-specific stimulation than it was before stimulation. Nonspecific stimulation led to a 2.6-fold increase in cytokine release per cell under the above assumption. Thus, Ag-specific function appears to be a durable property of TCR-transgenic T cells; moreover, their functional capacity can even be intensified, especially by repeated contact with endogenously processed Ag.

Cytotoxicity of CMV-TCR-transgenic T cells after Ag-specific vs nonspecific stimulation

Ag-specific cytotoxicity of CD8+ T cells is essential for the control of viral infection. After three rounds of stimulation, we investigated Ag-specific killing by expanded CMV-TCR-transgenic T cells in cocultures with pp65-positive and pp65-negative target cells (Fig. 7). After Ag-specific stimulation, NLV2-TCR-transduced T cells from CMV-negative donors efficiently lysed the autologous pp65-expressing mLCL even at very low E:T ratios. The HLA-mismatched pp65-expressing mLCL, pp65-negative mLCLs and BBLs were not or only very weakly recognized and killed by the T cells (Fig. 7A). After nonspecific stimulation, NLV2-TCR-transduced T cells were less efficient in killing the pp65-positive cells and showed an increased reactivity against the autologous as well as the HLA-mismatched control mLCLs. Mock-transduced PBMCs showed an EBV-specific or nonspecific killing pattern (Fig. 7A). Similar to the NLV2-TCR, transfer of the three other CMV-specific TCRs to primary T cells followed by stimulation resulted in efficient CMV-specific lysis of target cells (Fig. 7B). In each case, the specificity or intensity of lysis or both were superior after Ag-specific stimulation.
Phenotype of CMV-TCR-transgenic T cells during the stimulation process

CCR7 and L-selectin (CD62L) mediate homing of Ag-specific T cells to the lymph nodes, and their expression characterizes central memory T cells (42). We investigated the expression of these markers after TCR transfer (day 6) and Ag-specific or nonspecific stimulation (day 13, day 20). Expression of CCR7 and CD62L by NLV-TCR-expressing cells closely mirrored expression by the complete cell cultures (Fig. 8). The proportion of CCR7$^+$ and CD62L$^+$ cells decreased over time, but they accounted for $>15\%$ of CMV-TCR-positive or total T cells after specific expansion even at day 20.

These findings indicate that a proportion of CMV-TCR-transgenic T cells maintain their central memory phenotype after repeated Ag contact, which might contribute to the establishment of a TCR-transgenic CMV-specific T cell memory.

Discussion

In this study, we characterized CMV-specific T cells generated from CMV-seronegative donors by TCR transfer. Our intention was to investigate whether such T cells would be suitable for adoptive therapy of patients who suffer from CMV-related disease after allo-HSCT. Transfer of CMV-specific T cells might be ideal to
prevent and cure CMV disease, but for the combination CMV-negative donor/CMV-positive recipient (D+/R−) such cells are usually not available. However, for these patients, the risk of CMV disease is highest. D+/R− is usually the most frequent situation in allo-HSCT (5), but with decreasing CMV prevalence in the population, D+/R+ HSCTs will occur with increasing frequency.

The hypothesis underlying this study was that suitable CMV-specific TCRs are readily available within the memory T cell repertoire of CMV-seropositive donors. It can be expected that the TCR repertoire of CMV-specific memory T cells is shaped, at least in part, by the necessity to control CMV infection and to protect from disease. To select appropriate candidate T cell clones to

Table III. Total expansion and enrichment of CD8⁺ T cells expressing transgenic TCRs by Ag-specific or nonspecific stimulation

<table>
<thead>
<tr>
<th>CD8⁺ Multimer⁺ Cells</th>
<th>Expansion</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ag-specific stimulation</td>
<td>Nonspecific stimulation</td>
</tr>
<tr>
<td>Donor 1</td>
<td>Donor 2</td>
<td>Donor 1</td>
</tr>
<tr>
<td>Expansion</td>
<td>148</td>
<td>60</td>
</tr>
<tr>
<td>Enrichment</td>
<td>1.74</td>
<td>1.02</td>
</tr>
</tbody>
</table>

A Expansion of CMV-TCR-transgenic CD8⁺ T cells was calculated by dividing the absolute number of multimer⁺CD8⁺ T cells on day 20 by the absolute number of multimer⁺CD8⁺ T cells on day 4 (see also Fig. 4). Ag-specific or nonspecific stimulation was performed on days 6 and 13 (see Fig. 3A).

B Enrichment of CMV-TCR-transgenic CD8⁺ T cells was calculated by dividing the proportions of multimer⁺CD8⁺ T cells within total cells on day 20 by the proportions of multimer⁺CD8⁺ T cells within total cells on day 4 (see Fig. 4D).

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FIGURE 5. CMV-Ag-specific release of effector cytokines by CMV-TCR-transduced PBMCs after three rounds of stimulation. PBMCs derived from CMV-seronegative donors (A, donor 2; B and C, donor 3) were retrovirally transduced with different CMV-specific TCRs, or mock-transduced as indicated. Three rounds of Ag-specific stimulation were performed with nonspecific stimulation performed on days 6 and 13 (see Fig. 3A). The TCR repertoire of CMV-specific memory T cells is shaped, at least in part, by the necessity to control CMV infection and to protect from disease. To select appropriate candidate T cell clones to

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increase in Ag-specific effector functions (cytotoxicity and cytokine secretion) in reaction to target cells was analyzed before (day 6) and after (day 25) three rounds of Ag-specific (left) or nonspecific (right) stimulation. Target cells included autologous and HLA-mismatched pp65-expressing mLCLs and control mLCLs. Cytokine release per multimer-positive cell was calculated by dividing the total amount of cytokine detected in the reaction supernatant by the number of total T cells per reaction multiplied by the proportion of multimer-positive cells assessed at the nearest available time point: 4.23% on day 4 (before stimulation); 11.2% on day 28 (after specific stimulation); 0.79% on day 28 (after nonspecific stimulation).

To investigate the structure of the corresponding TCR-peptide-MHC complex, we characterized two previously undescribed TCRs with different HLA restrictions, one recognizing the HLA-A*0101-restricted epitope YSE and one specific for the HLA-B*3501-restricted epitope IPS. We found that retroviral transfer of all these CMV-specific TCRs resulted in TCR-transgenic T cells which exhibited a wide spectrum of desirable functional capacity of multimer-positive cells even appeared to increase over time (Fig. 6). Furthermore, no stimulation of TCR-transduced T cells, cell sorting or depletion was required to obtain T cells displaying Ag-specific function at very convincing levels as early as 6 days after TCR transfer (Fig. 2).

These results prompt us to suggest that a simple protocol consisting of TCR transfer to PBMCs, without further expansion or selection steps, will produce CMV-TCR-transgenic T cell populations suitable for immediate use in T cell transfer therapy. Care should be taken to keep the total number of allogenic T cells transferred to a patient low enough to minimize the probability of graft-versus-host disease, for example, <10⁶ cells/kg for HLA-matched donor-recipient pairs (43), because allogeneic or originally tolerant self-reactive T cells might be present in the TCR-transduced T cell preparation. Such potentially harmful T cells might be activated by the anti-CD3 treatment preceding transduction, or by means of a transgenic CMV-specific TCR coexpressed on the same cell and recognizing its target Ag in the patient. However, as long as T cell therapy is performed early enough in a pre-emptive situation, low numbers of CMV Ag-specific T cells may be sufficient to avert disease (9). We consider it likely that Ag-driven T cell expansion in the CMV-infected HSCT patient, possibly favored by lymphopenia (1), will be at least as effective in producing sufficient numbers of specific effector T cells than any further expansion in vitro. The considerable proportions of CMV-TCR-transgenic T cells positive for the central memory markers CCR7 and CD62L (Fig. 8), which were largely retained after Ag contact in vitro, further suggest that TCR-transgenic cells will be able to form an effective CMV-specific memory in vivo.

Diverse strategies to obtain CMV-specific T cells for adoptive therapy after D−/R+ HSCT might be taken into consideration. CMV-specific T cells could be obtained directly from a third unrelated CMV-seropositive donor. In this context, third-party-derived EBV-specific T cells had very good therapeutic effects in solid organ transplant recipients (44). However, allogeneic T cells might be rapidly rejected, hampering long-term antiviral protection. Therefore, T cells derived from the HSCT donor are to be preferred for adoptive therapy. Another alternative to CMV-TCR transfer would be in vitro priming and expansion of rare naive CMV-specific T cells from the donor. Remarkably, with a small proportion of CMV-seronegative donors it has been possible to generate CMV-specific T cells by in vitro priming (45–47). However, we expect it to be very difficult to translate these observations into feasible clinical procedures, because the precursor frequency of naive CMV-specific CD⁸⁺ T cells in CMV-seronegative individuals, estimated from general considerations on TCR diversity (48), is unlikely to be above 1 in 10⁶ naive T cells. Therefore, transfer of CMV-specific TCRs to primary T cells will be the easiest and most efficient method for adoptive therapy of CMV-related complications after HSCT with CMV-seronegative donors.

By using CMV-specific TCRs of various HLA restrictions, population coverage can be optimized. With the selected HLA-A*0101-, HLA-A*0201-, and HLA-B*3501-restricted CMV-specific TCRs, at least 70% of Europeans can be covered, HLA-A*0201 being the most frequent and HLA-A*0101 the second most frequent HLA alloype. Obviously, further extension of this TCR repertoire to cover additional HLA allootypes is highly desirable.
is possible to transfer HLA-B7-restricted (49) or HLA-A2-restricted (27, 50) pp65-specific TCRs to H9253/H9254 T cells (27), H9251/H9252 T cells (27), or in vitro-differentiated hemopoietic precursors (50), and showed that pp65 peptide-loaded targets (27, 49, 50) and LCLs transduced with pp65 (49, 50) were recognized. In these studies, CMV served as a model specificity to establish principles of TCR transfer. Aiming at the development of a CMV-specific T cell therapy in an HSCT context, our study extends previous findings with respect to the following points: we identified and transferred CMV-specific TCRs restricted through the frequent HLA alleles A*0101 and B*3501; we used a facilitated method of TCR transfer that does not require cell sorting and the expression of heterologous marker genes in the transduced T cells; we performed CMV-specific TCR transfer with an extended panel of donors and analyzed T cell reactivity against target cells of various HLA types; in accordance with our intended therapeutic goal, we used donors who were CMV-negative, excluding the possibility that endogenous CMV-specific T cells might contribute to the observed effects; we showed that CMV-TCR-transgenic T cells specifically and continuously expand in response to repeated challenge with endogenously presented Ag, while fully maintaining their CMV-specific effector functions.

It was previously described that different CMV-specific TCRs recognizing the HLA-A*0201-restricted NLV epitope significantly differ in their efficiency in conferring CMV-specific function to PBMC cultures (27). This observation suggests that only some CMV-specific TCRs may be suitable for clinical application. Similarly, we observed different surface expression levels for different TCRs (Fig. 2, A–C). TCR expression levels appeared to depend on their HLA restriction: the two HLA-A*0201-restricted TCRs, recognizing the NLV epitope, were expressed at higher levels than the HLA-A*0101- and HLA-B*3501-restricted TCRs. This effect was observed both in primary T cells and TCR-deficient J76 cells and therefore appears to be related to “intrinsic properties” (27) of the TCRs. Additionally, a difference in the expression levels among the two HLA-A*0201-restricted TCRs was observed only in primary T cells, but not in J76 cells, which implies that interference by endogenous TCRs might play a role here. Despite these differences early after transduction, surface expression as well as specific function of the different TCRs became rather similar after repeated Ag-specific stimulation (Figs. 4, 5, and 6). Because we observed that the lower initial transduction rates of the IPS- and YSE-TCRs were compensated by a stronger Ag-driven enrichment, we speculate that all four TCRs investigated in this study will be similarly efficient in adoptive therapy.

In our study, we performed retroviral transfer of unmodified TCR chain genes. Modifications of the TCR for increased surface expression or reduced TCR chain cross-pairing have been...
arsenal of specific antiviral functions. We believe that such mechanisms are essential for rapidly preparing TCR-transgenic T cells equipped with an antiviral repertoire. We demonstrated that these TCRs can be used to efficiently recognize endogenously processed viral Ag. We further showed that these TCRs are characterized by efficient recognition of HLA class I-restricted Ags. In particular, we observed that these TCRs can be used to rapidly prepare TCR-transgenic T cells equipped with an arsenal of specific antiviral functions. We believe that such TCR-transgenic T cells qualify as candidates for a successful clinical application in HSCT patients with a CMV-negative donor, a situation of considerable and potentially increasing clinical importance.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


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**Figure 8**

Central memory markers on CMV-TCR-transgenic T cells during stimulation. PBMCs derived from CMV-seronegative donor 1 were retrovirally transduced with the CMV-specific NLV2-TCR or NLV3-TCR followed by Ag-specific or nonspecific stimulation. The frequency of CCR7-positive T cells was assessed at days 6, 13, and 20 after TCR transfer by staining with CCR7-specific Ab and NLV tetramer and subsequent flow cytometric analysis. The proportion of CCR7-positive cells within the NLV tetramer-positive population (■) as well as the proportion of CCR7-positive cells within the total T cell population (□) is shown. Similarly, the frequency of CD62L+ T cells was assessed at days 6, 13, and 20 after TCR transfer by staining with CD62L-specific Ab and NLV tetramer followed by flow cytometric analysis. The proportion of CD62L+ cells within the NLV tetramer-positive population (■) as well as the proportion of CD62L+ cells within total lymphocytes (□) is shown.


